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Critical Role of Complement and Viral Evasion of Complement in Acute, Persistent, and Latent γ-Herpesvirus Infection

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Summary

Several γ-herpesviruses encode homologs of host regulators of complement activation (RCA) proteins, suggesting that they have evolved immune evasion strategies targeting complement. We evaluated the role of complement factor C3 (C3) and the murine γ-herpesvirus 68 (γHV68) RCA protein in viral pathogenesis. Deletion of the γHV68 RCA protein decreased virulence during acute CNS infection, and this attenuation was specifically reversed by deletion of host C3. The γHV68 RCA protein was also important for persistent viral replication and virulence in IFNγR$^{-/-}$ mice. In addition, C3 played a role in regulating latency, but this was not counteracted by the γHV68 RCA protein. We conclude that complement is a key host defense against γ-herpesvirus infection and that γ-herpesviruses have evolved an immune evasion strategy that is effective against complement-mediated antiviral responses during acute but not latent infection.

Introduction

Complement plays a protective role in the acute host response against several viruses (Lewis et al., 1986; Rundell and Betts, 1982; Davis-Poynter and Farrell, 1996; Hicks et al., 1978; Hirsch et al., 1980a, 1980b; Leddy et al., 1977), and therefore it is not surprising that viruses have developed strategies to block the effects of complement. The host uses a family of proteins termed regulators of complement activation (RCA proteins) containing multiple copies of an approximately 60 amino acid domain called the short consensus repeat (SCR) to prevent damage to host cells by activated complement (Liszewski et al., 1996; Reid and Day, 1989). Viral strategies for evading complement include incorporation of host RCA proteins into virions, encoding secreted or cell surface expressed RCA protein homologs, or expression of structurally unrelated complement regulatory proteins (Saifuddin et al., 1995; Spear et al., 1995; Takefman et al., 1998; Rother et al., 1995; Hirsch et al., 1980b).

γ-herpesviruses establish life-long infection in the majority of humans, with diseases including cancer occurring especially in immunocompromised people. Several oncogenic γ-herpesviruses, including the human Kaposi's sarcoma-associated herpesvirus (KSHV), the primate herpesvirus saimiri (HVS), and the murine γHV68, encode RCA protein homologs (Russo et al., 1996; Virgin et al., 1997; Rother et al., 1994; Fodor et al., 1995; Albrecht and Fleckenstein, 1992; Kapadia et al., 1999), but their role in infection is unknown. The γHV68 and HVS RCA proteins inhibit complement activation in vitro at the level of C3 and/or C4 deposition (Virgin et al., 1997; Kapadia et al., 1999; Rother et al., 1994; Fodor et al., 1995; Albrecht and Fleckenstein, 1992). The γHV68 RCA protein has both a soluble isoform, which inhibits C3 activation via both the classical and alternative pathways, as well as an isoform expressed on infected cell surfaces and virions (Kapadia et al., 1999; Gangappa et al., submitted).

The γHV68 RCA protein is not essential for γHV68 replication in cultured cells (Adler et al., 2000), suggesting that the virus encodes this protein to evade the host response to infection rather than to assist in viral replication per se. The conservation of RCA proteins in several γ-herpesviruses also suggests the hypothesis that complement plays a protective role in host defense against these viruses. However, since proteins with SCRs can have functions other than complement regulation (del Mar et al., 1998; Isaacs et al., 1992b; Martinez-Pomares et al., 1993; Engelstad and Smith, 1993; Wolffe et al., 1993; Takahashi-Nishimaki et al., 1991; Herrera et al., 1998; Mathew et al., 1998; Laquerre et al., 1998; Sears et al., 1991; Lubinski et al., 1998), it is possible that the function of the γ-herpesvirus SCR-containing RCA proteins is not related to complement.

To address the hypothesis that complement is an important host defense against γ-herpesvirus infection, we assessed the role of C3 and the γHV68 RCA protein in three components of γHV68 infection in vivo: (1) viral replication and virulence during acute infection, (2) viral replication and virulence during persistent infection of immunocompromised mice, and (3) establishment of and reactivation from viral latency. γHV68 provides a useful model for these studies since it is closely related to Epstein Barr virus (EBV), HVS, and KSHV (Efstathiou et al., 1990a, 1990b; Virgin et al., 1997) but infects laboratory mice (Mistrikova and Blaskovic, 1985; Rajcani et al., 1985; Blaskovic et al., 1980; Terry et al., 2000; Sunil-Chandra et al., 1992).

During acute infection, γHV68 causes pneumonia or meningoencephalitis depending on the route of inoculation (Terry et al., 2000; Rajcani et al., 1985). Host resistance to acute infection involves T cells, IFNαβ, chemokines, and STAT1 (Kulkarni et al., 1997; Ehtisham et al., 1993; Dutia et al., 1999). During chronic infection, γHV68

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can establish latency or, in immunocompromised (e.g., IFN$\gamma^{-/-}$ or IFNγ receptor$^{-/-}$) mice, undergo persistent replication. Persistent replication is associated with severe disease including splenic fibrosis and vasculitis restricted to the great elastic arteries (Clambey et al., 2000; Usherwood et al., 2000; Weck et al., 1997; Dal Canto and Virgin, 1999, 2000; Dutia et al., 1997; Dal Canto et al., 2000; Tibbetts et al., 2002). Persistent replication is regulated by T cells, B cells, and IFNγ (Usherwood et al., 1996, 2000; Weck et al., 1999a; Tibbetts et al., 2002; Christensen et al., 1999; Kim et al., 2002) and is a viral process that is distinct from acute replication since it requires different viral genes (Gangappa et al., 2002). γHV68 establishes latency in B cells, macrophages, and dendritic cells (Weck et al., 1996, 1999b; Flano et al., 2000; Sunil-Chandra et al., 1992). γHV68 latency is regulated by T cells, B cells, IFNγ, and perforin (Weck et al., 1999a; Topham et al., 2001; Gangappa et al., 2002; Flano et al., 1999; Kulkarni et al., 1997; Usherwood et al., 2000).

Two sets of considerations suggested that complement and the γHV68 RCA protein might be important in specific in vivo settings. First, CNS cells can express most complement components, particularly after cytokine stimulation or viral infection (Johnston et al., 2001; Morgan and Gasque, 1996; Speth et al., 2002), and mRNAs for multiple complement components are upregulated in the CNS of virus-infected mice (Johnston et al., 2001; Terry et al., 2000). We therefore reasoned that complement might play a role in controlling meningoencephalitis observed after intracranial (i.c.) inoculation of γHV68 (Terry et al., 2000; van Berkel et al., 2002). Second, complement regulates multiple aspects of B cell biology (Ochsenbein et al., 1999; Carroll and Fischer, 1997; Prodeus et al., 1997, 1998). Since γHV68 establishes latent infection in B cells (Weck et al., 1996, 1999a, 1999b; Flano et al., 2000; Sunil-Chandra et al., 1992) and B cells regulate γHV68 latency (Weck et al., 1999a), we considered it possible that complement might be important during latent γHV68 infection.

To determine the role of complement and the γHV68 RCA protein in vivo, we utilized γHV68 RCA protein mutant viruses and mice lacking C3 or complement factor B. We found that complement plays a crucial role during acute, persistent, and latent γHV68 infection. The γHV68 RCA protein effectively counteracts some but not all of these actions of complement in vivo.

Results

Disruption of the γHV68 RCA Protein Open Reading Frame

γHV68-RCAstop and a control marker rescue virus (γHV68-RCAstop.MR) were constructed by homologous recombination (Figure 1A). Their genomic structure (probe A in Figure 1B) and the absence of deletions at the left end of the genome (Clambey et al., 2000; Van Dyk et al., 2000) (probe B in Figure 1B) were confirmed by Southern blot analysis. γHV68-RCAstop infected cells did not express the three known isoforms of the γHV68 RCA protein (Kapadia et al., 1999) (Figure 1D). Expression of γHV68 RCA protein was restored in γHV68-RCAstop.MR (Figure 1D). Insertion of a translational stop codon in the γHV68 *RCA* gene at bp 10,023 could result in the generation of an $\sim$50 amino acid truncated protein with an intact signal sequence (Figure 1C). However, we did not detect truncated forms of the RCA protein on Western blots (Figure 1D and data not shown), and supernatants from cells infected with γHV68-RCAstop did not block C3 deposition on activated zymosan particles (Kapadia et al., 1999) (data not shown). Therefore, the RCAstop mutation eliminated expression of functional RCA protein. Insertion of the RCAstop mutation did not change transcript levels from adjacent genes *M4* or *gene 6* in infected fibroblasts (Figure 1E), indicating that phenotypes of the RCAstop mutation were not attributable to unanticipated effects of the RCAstop mutation on transcription of adjacent genes.

The γHV68 RCA Protein Is Not Essential for Viral Replication during Acute Infection

Consistent with previous findings (Adler et al., 2000), γHV68-RCAstop replicated normally in cultured NIH 3T12 fibroblasts (Figure 1F), demonstrating that the gene is not essential for viral replication in vitro. To determine whether the γHV68 RCA protein is involved in replication during acute infection in vivo, adult B6 mice were infected with γHV68-RCAstop and wild-type γHV68. In normal B6 mice γHV68-RCAstop titers were modestly decreased on day 4 (2.5-fold, $p = 0.0084$) and day 9 (2.9-fold, $p = 0.0833$) postinfection in the spleen (Figure 2A) but not liver or lung after i.p. inoculation (data not shown). In contrast, no defect in acute splenic replication of γHV68-RCAstop was observed in C3$^{-/-}$ mice (Figure 2B). We further assessed γHV68-RCAstop growth in 21-day-old CD-1 mice after i.p. inoculation. γHV68-RCAstop titers were decreased 2.3- to 3.8-fold in the lung ($p = 0.0005$, Figure 2C) and spleen ($p = 0.0119$, Figure 2C). These data indicate that complement and the γHV68 RCA protein may play a modest role in controlling acute viral replication in spleen, liver, and lung of immunocompetent mice after i.p. inoculation. However, the physiologic significance of such small changes in viral titer is not clear.

The γHV68 RCA Protein Is a Virulence Factor during Acute and Chronic Infection

We next examined the role of the γHV68 RCA protein in two established models of γHV68 virulence: acute lethal meningoencephalitis in weanling wild-type mice and mortality during persistent infection of IFNγR$^{-/-}$ mice. γHV68-RCAstop was less virulent than either γHV68 or γHV68-RCAstop.MR after i.c. inoculation (Figure 2D). The LD_{50} was $\sim$1 pfu for wild-type γHV68 and γHV68-RCAstop.MR, while the LD_{50} for γHV68-RCAstop was approximately 100-fold higher ($p < 0.0001$ for the 10 and 100 pfu dose, Figure 2D). γHV68-RCAstop was also significantly attenuated during persistent infection of IFNγR$^{-/-}$ mice ($p = 0.0249$, Figure 2E). The decrease in virulence in acutely infected weanling mice and IFNγR$^{-/-}$ mice was due to the stop mutation in the γHV68 RCA gene since virulence was fully restored in γHV68-RCAstop.MR (Figures 2D and 2E). These data demonstrate that the γHV68 RCA protein is a virulence factor during acute and chronic infection.

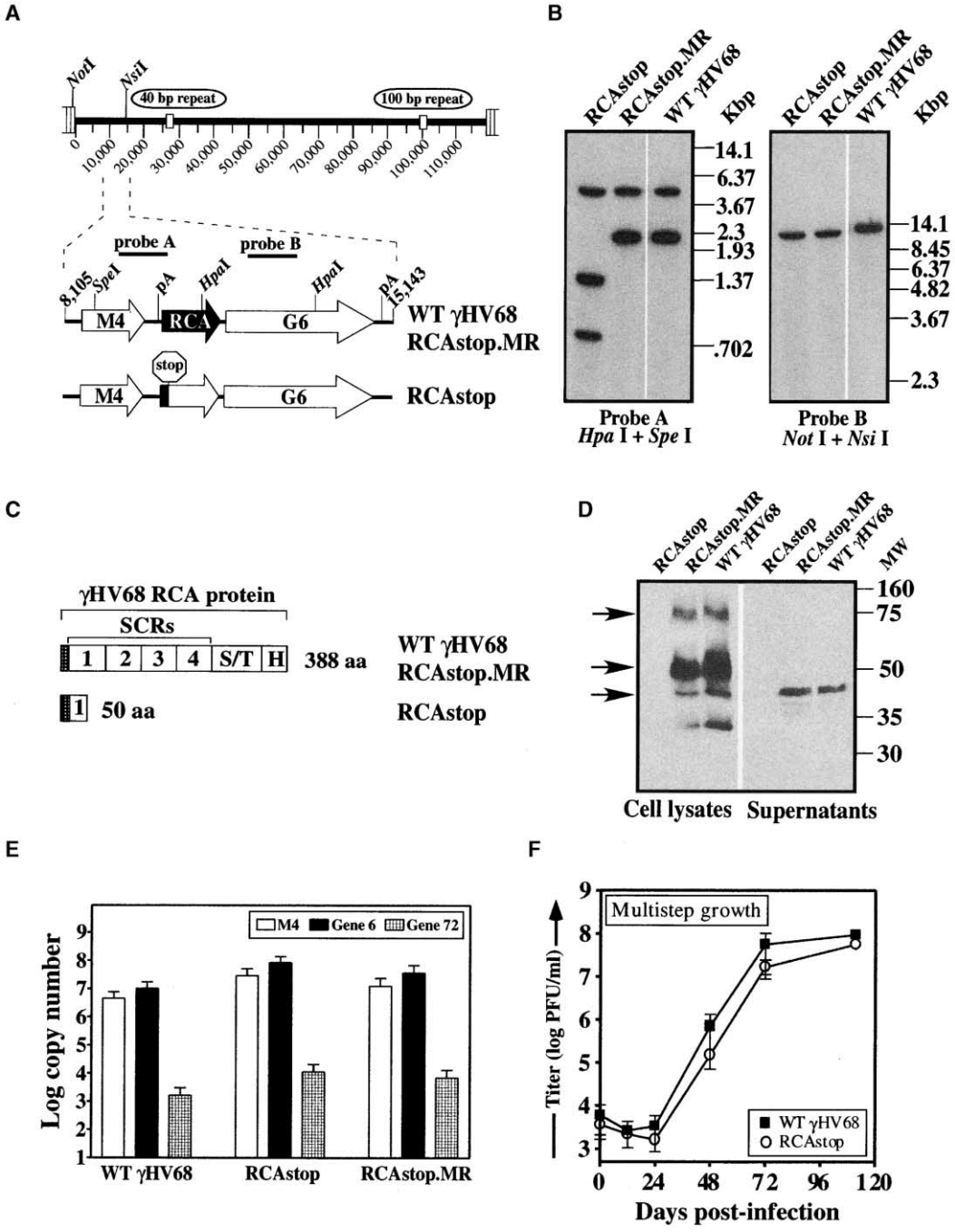

Figure 1. Construction and Characterization of the γHV68-RCAstop Mutant Virus

(A) Genomic structure of wild-type (WT) γHV68, γHV68-RCAstop, and marker rescue (MR) viruses. Probe A is the MluI-MfeI fragment from bp 7949 to 10,584 of the γHV68 genome (Virgin et al., 1997). Probe B is the HindIII-EcoRV fragment from bp 11,100 to 14,026 of the γHV68 genome (Clambey et al., 2000; Van Dyk et al., 2000; Virgin et al., 1997). pA, polyadenylation signals. Adjacent ORFs (M4 and G6) are shown (Virgin et al., 1997).

(B) Southern blot analysis using probe A with HpaI+SpeI digestion (left panel) and probe B with NotI and NsiI digestion (right panel) of γHV68 DNA. Note the 4.041, 1.357, and 0.758 kb fragments with γHV68-RCAstop DNA, as compared to the 4.041 and 2.114 kb fragments with wild-type and marker rescue viruses. Probe B did not detect smaller fragments in either wild-type γHV68, γHV68-RCAstop, or γHV68-RCAstop.MR viruses, indicating that no large deletions occurred at the left end of the genome (right panel).

(C) Sizes of putative truncated γHV68 RCA protein products generated by the mutant viruses.

(D) Western blot analysis to detect expression of the different γHV68 RCA protein isoforms (MW 60–65, 50–55, and 40–45 kDa).

(E) Real-time RT-PCR analysis of *M4* and *gene 6* transcription in NIH 3T12 cells infected with either wild-type γHV68, γHV68-RCAstop, or γHV68-RCAstop.MR. *Gene 6* and *gene 72* (v-cyclin) transcription was analyzed in parallel as controls. Shown are mean ± SEM of pooled data from four independent experiments.

(F) Multistep growth of wild-type γHV68 and γHV68-RCAstop.

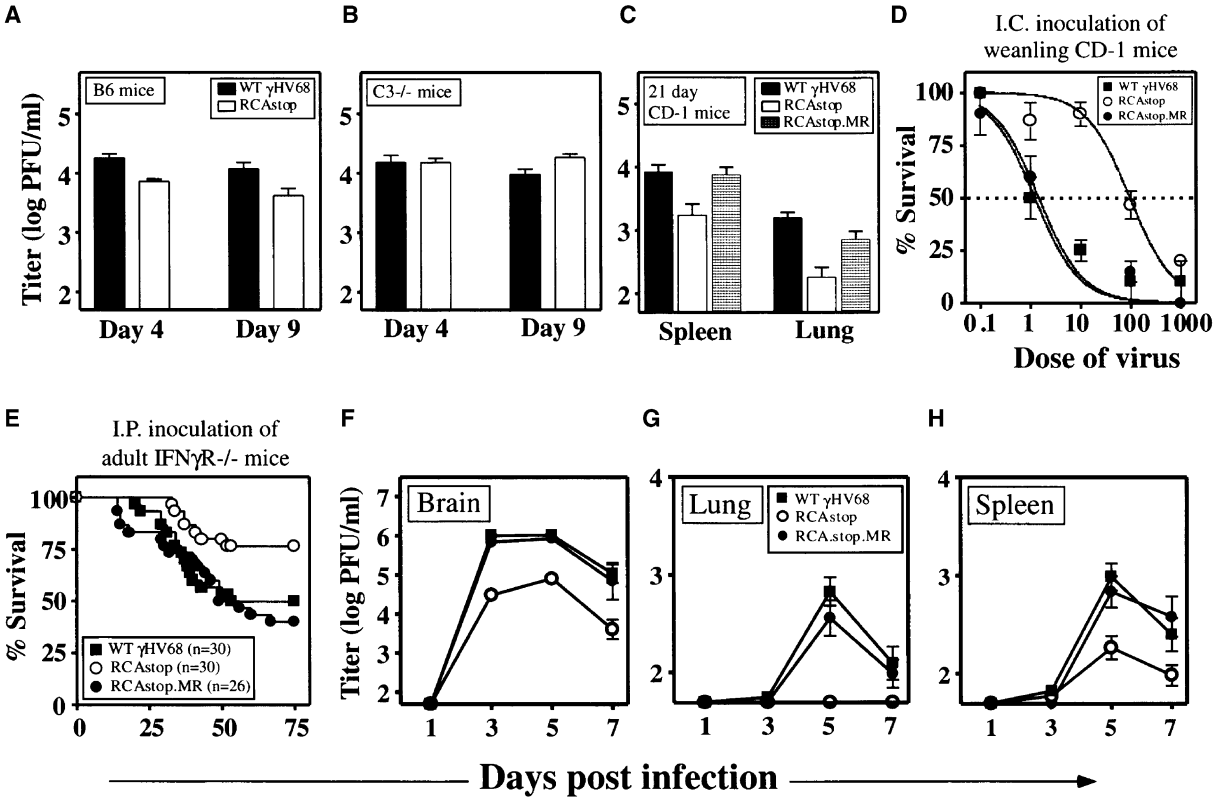

Figure 2. Growth and Virulence of γHV68-RCAstop

(A and B) Acute splenic viral titers at days 4 and 9 after i.p. inoculation in adult B6 and C3$^{-/-}$ mice.

(C) Acute viral splenic and lung titers in 21-day-old wild-type CD-1 mice after i.p. inoculation. Mice were sacrificed, and organs were harvested 5 days postinfection. For (A)–(C), shown are mean ± SEM of pooled data from two independent experiments (at least ten mice total per condition).

(D) Survival of weanling CD-1 mice after i.c. inoculation of γHV68-RCAstop. Mice were infected with the indicated virus, and survival was assessed after 14 days of infection. Each point represents between 20–30 mice per dose for each virus from at least three independent experiments.

(E) Survival of adult IFNγR$^{-/-}$ mice infected with γHV68-RCAstop, γHV68-RCAstop.MR, and wild-type γHV68. n, number of mice.

(F–H) Viral titers after i.c. inoculation of 21-day-old CD-1 mice with 100 pfu of virus. Shown are mean ± SEM of pooled data from at least two independent experiments (ten mice per virus per time point).

Critical Role for the γHV68 RCA Protein in Meningoencephalitis during Acute Infection

The most significant (~100-fold) attenuation attributable to loss of the γHV68 RCA protein was observed after i.c. inoculation of weanling mice (Figure 2D). In this setting, attenuation of γHV68-RCAstop was associated with an ~27-fold decrease in viral growth in the CNS compared to γHV68 or γHV68-RCAstop.MR ($p = 0.0001$, Figure 2F). γHV68-RCAstop also failed to efficiently spread to and replicate in the lung ($p = 0.0377$, Figure 2G) and spleen ($p = 0.0035$, Figure 2H) after i.c. inoculation. To assure that the critical role for the γHV68 RCA protein in meningoencephalitis was not specific to CD-1 mice, we confirmed that γHV68-RCAstop is attenuated after i.c. inoculation of 129 x B6 F1 mice (see below) and that growth of γHV68-RCAstop was significantly decreased in brains, spleen, and lungs of inbred 129 x B6 F1 mice after i.c. inoculation (data not shown). It is interesting to note that the effect of complement on γHV68 pathogenesis was greatest in the CNS where it is generally considered that complement levels are low in the absence of inflammation.

Immunofluorescence staining revealed viral antigen-positive cells in the meninges and the brain parenchyma of wild-type γHV68-infected mice 5 days postinfection (Figures 3A–3C). Consistent with the observed lower viral titers (Figure 2F), staining was significantly decreased in γHV68-RCAstop-infected mice compared to wild-type γHV68-infected mice (compare Figures 3A to 3G and Figures 3B to 3H). We detected the γHV68 RCA protein in the meninges of infected mice (Figures 3D and 3E). While staining with the polyclonal anti-γHV68 antibody revealed viral proteins in both the cytoplasm and in the nucleus of virus-infected parenchymal cells (Figures 3C and 3I), the γHV68 RCA protein was restricted to the cytoplasm and plasmalemma of infected CNS cells (Figures 3E, inset, and 3F). The potential physiologic role of cytoplasmic γHV68 RCA protein is not known.

In mice infected with wild-type γHV68, the number of infected brain parenchymal cells increased between 5 and 7 days postinfection (Figure 4). The γHV68 RCA protein played a key role in promoting parenchymal invasion since γHV68-RCAstop infected 10-fold fewer pa-

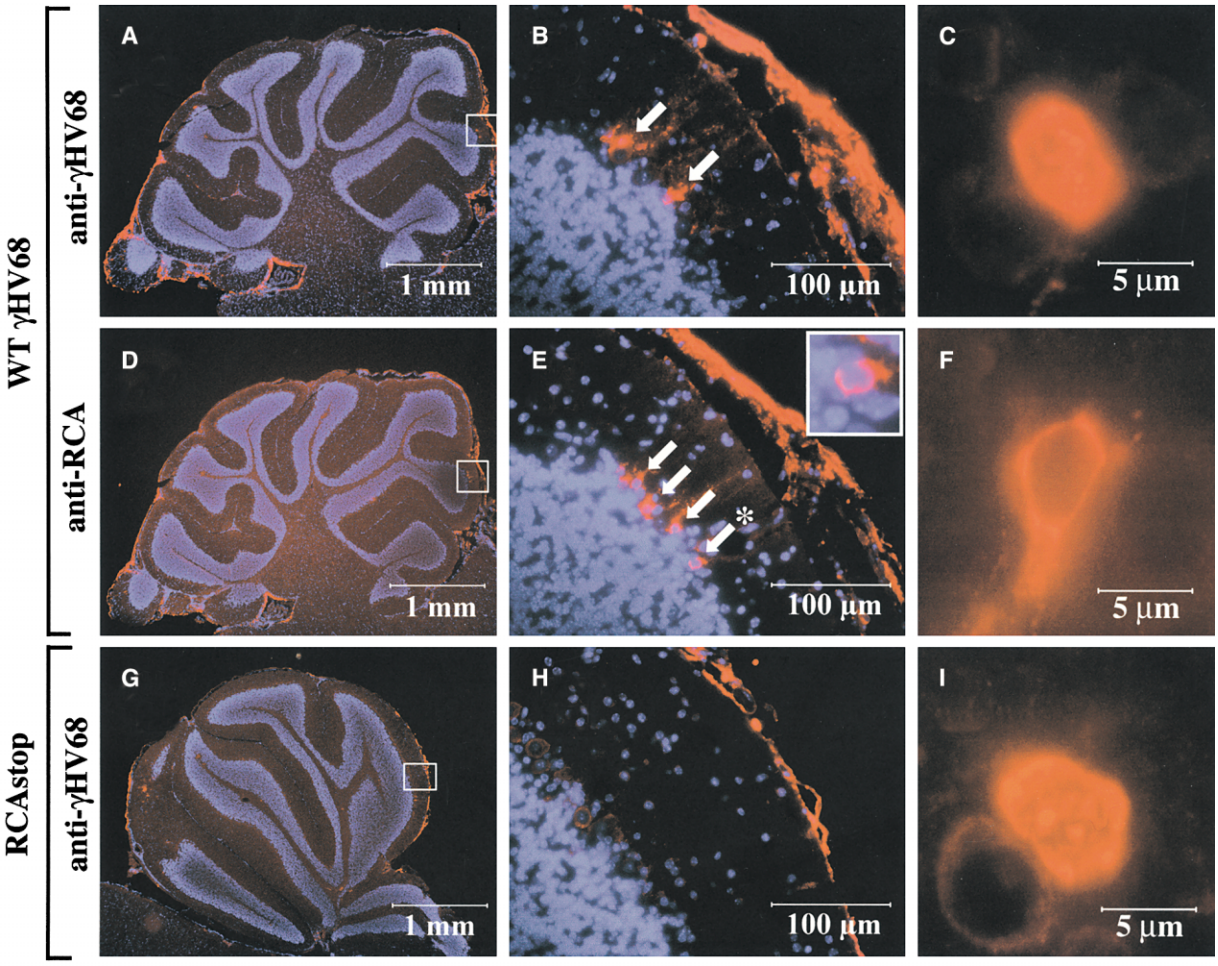

Figure 3. γHV68 Antigen in the Brains of Mice with Meningoencephalitis

Shown are saggital sections of the cerebellum from CD-1 mice infected for 5 days with wild-type γHV68 (A–F) or γHV68-RCAstop (G–I). Sections were immunostained using either anti-γHV68 polyclonal antibody (A–C and G–I) or anti-γHV68 RCA polyclonal antibody (D–F). (B), (E), and (H) are magnifications of the boxed regions in (A), (D), and (G). Arrows denote viral antigen-positive cells. In (E), the asterisk denotes the cell shown in the inset. No antigen was detected with the preimmune serum or in mock-infected mice (data not shown). These images are representative of eight to ten mice per group. FACS analysis demonstrated that the polyclonal anti-γHV68 antibody bound with similar efficiency to NIH 3T12 cells infected with either γHV68-RCAstop or wild-type γHV68 (data not shown). Thus, the decreases in meningeal viral antigen and the number of infected parenchymal cells in mice infected with γHV68-RCAstop was not an artifact of the γHV68 RCA protein being the dominant antigen recognized by the polyclonal anti-γHV68 antibody.

renchymal cells than wild-type γHV68 7 days after infection ($p = 0.002$, Figure 4E).

C3-Mediated Resistance Is the Physiologic Target of the γHV68 RCA Protein In Vivo

Since SCR-containing proteins can have activities other than complement regulation, we evaluated whether the role of the γHV68 RCA proteins in virulence was due to regulation of complement. We therefore applied a genetic test (host complementation), previously used to prove that the HSV ICP34.5 protein targets PKR-dependent processes in vivo (Leib et al., 2000) and that the HSV gC protein acts in vivo to target complement (Lubinski et al., 1998, 1999; Friedman et al., 1996). In this test, removal of the host target (C3) of a viral virulence gene (γHV68 RCA protein) should restore virulence to a viral mutant in the virulence gene (γHV68-RCAstop), and this restoration of virulence should be specific to both the viral gene and the host gene involved.

If the γHV68 RCA protein effectively inhibits C3-mediated host defense, then the presence or absence of C3 in the host should make no difference for viral virulence as long as the γHV68-RCA protein is present. Consistent with this, there was no difference in kinetics of lethality or the percentage of mice that die between CD-1 mice, wild-type 129 x B6 F1 mice, $C3^{-/-}$ mice, or factor $B^{-/-}$ mice infected with wild-type γHV68 (Figures 5A–5D). 129 x B6 F1 mice were included as a control for $C3^{-/-}$ and factor $B^{-/-}$ mice, which are on a mixed 129 x B6 background (Matsumoto et al., 1997).

γHV68-RCAstop was significantly attenuated in virulence in weanling CD-1 mice ($p = 0.0095$, Figure 5A).

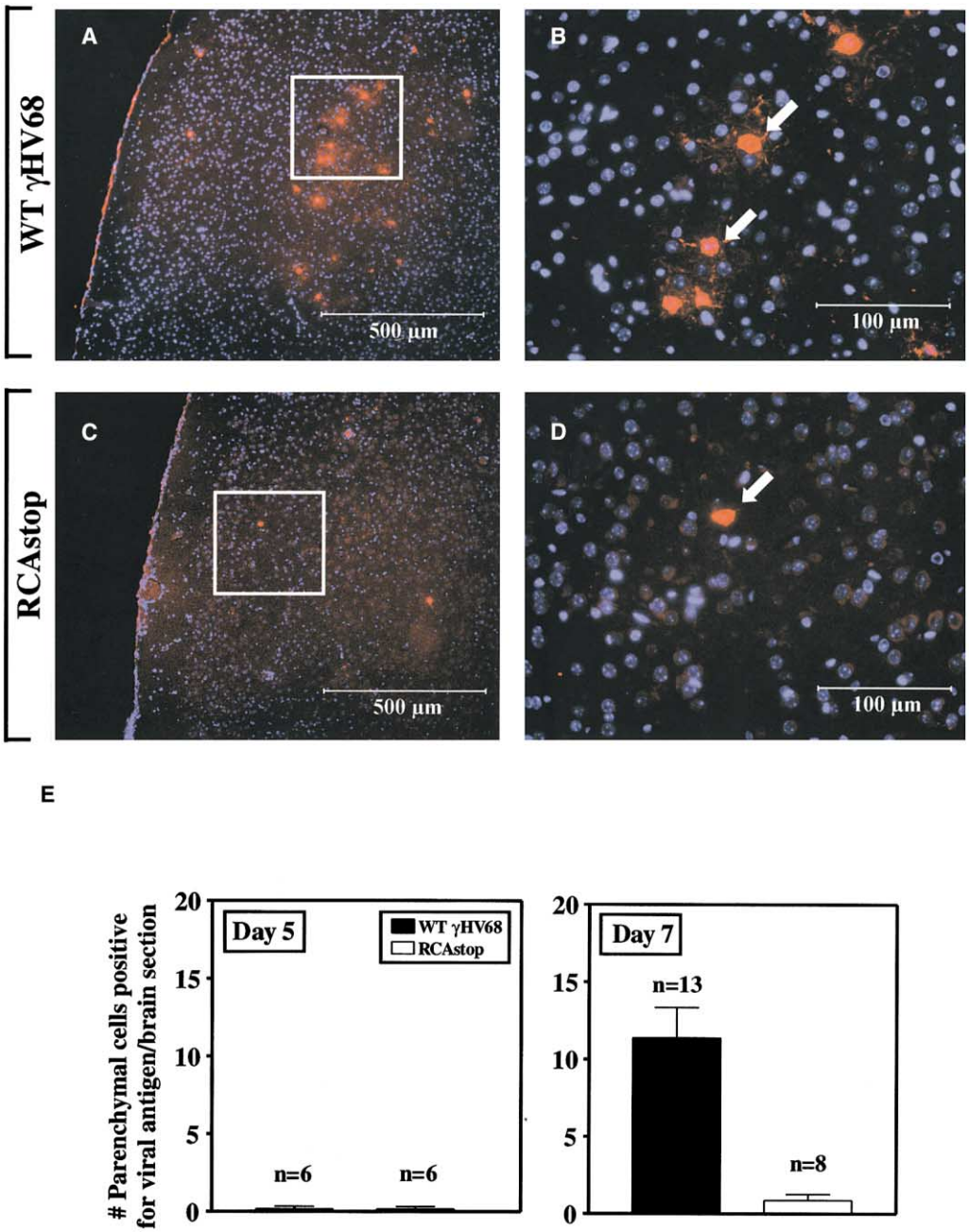

Figure 4. The Frequency of Virus-Positive Parenchymal Cells 7 Days Postinfection

Virus antigen-positive cells in the parenchyma of CD-1 mice infected with wild-type γHV68 (A and B) or γHV68-RCAstop (C and D). (B) and (D) are magnifications of the boxed regions in (A) and (C). Arrows denote viral antigen-positive cells. In (E), the number of parenchymal cells staining positive for viral antigen was quantitated by three individuals counting antigen-positive cells in a blinded manner (see Experimental Procedures). The numbers on top of the bars represent the number of mice per group.

As expected from studies in CD-1 mice (Figures 2D and 5A), γHV68-RCAstop was significantly attenuated in 21-day-old 129 x B6 F1 mice ($p < 0.0001$, Figure 5B). Consistent with the γHV68 RCA protein targeting C3-dependent host resistence, deletion of C3 in the host restored virulence of γHV68-RCAstop to wild-type levels ($p = 0.0102$, Figure 5C).

To determine the specificity of the restoration of γHV68-RCAstop virulence by deletion of C3, we performed two additional experiments. First, we infected $C3^{-/-}$ and wild-type mice with γHV68-Cyclin.LacZ (Van Dyk et al., 2000). γHV68-Cyclin.LacZ was attenuated after i.c. inoculation of both 129 x B6 F1 mice ($p < 0.0001$, Figure 5B) and $C3^{-/-}$ mice ($p = 0.0025$, Figure 5C). Furthermore, deletion of factor B from the host did not restore virulence of γHV68-RCAstop (Figure 5D).

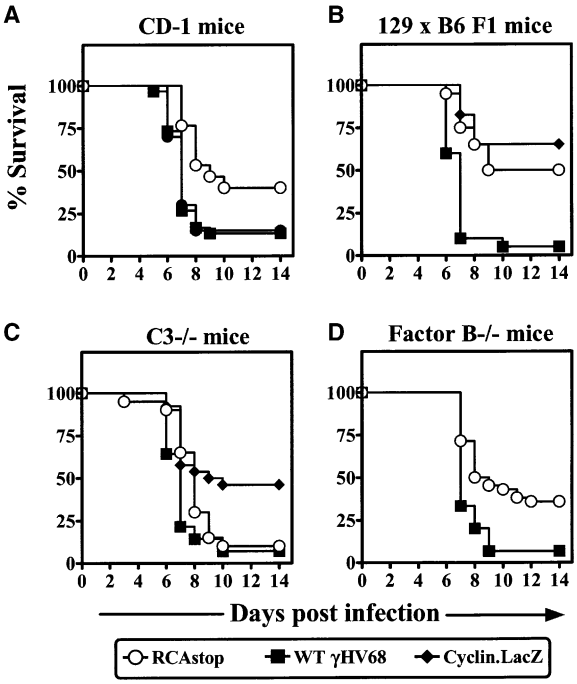

Figure 5. In Vivo Host Complementation of γHV68-RCAstop Attenuation

Twenty-one-day-old wild-type CD-1 mice (A), 129 x B6 F1 mice (B), $C3^{-/-}$ mice (C), and factor $B^{-/-}$ mice (D) were infected with 100 pfu of virus, and survival was plotted versus time postinfection. This data is pooled from at least three independent experiments with a total of 30 CD-1 mice, 14 $C3^{-/-}$ mice, 15 factor $B^{-/-}$ mice, and 20 129 x B6 F1 mice infected with wild-type γHV68; 30 CD-1, 20 $C3^{-/-}$ mice, 42 factor $B^{-/-}$ mice, and 20 129 x B6 F1 mice infected with γHV68-RCAstop; and 26 $C3^{-/-}$ mice and 20 129 x B6 F1 mice infected with Cyclin.LacZ.

Thus, restoration of γHV68-RCAstop virulence in $C3^{-/-}$ mice is specific for both the viral gene (γHV68 RCA) and the host gene (C3 versus Factor B) involved. We therefore conclude that the γHV68 RCA protein specifically targets C3-dependent host resistance in vivo.

The γHV68 RCA Protein Plays a Role in Persistent Viral Replication in Immunocompromised IFNγR$^{-/-}$ Mice

In addition to virulence during acute infection, the γHV68 RCA protein played a role in virulence during persistent replication of IFNγR$^{-/-}$ mice (Figure 2E). Consistent with prior reports (Tibbetts et al., 2002; Gangappa et al., 2002; Presti et al., 1998; Weck et al., 1997; Dal Canto et al., 2000), persistent replication was not detected in normal mice at 16 days after infection (Figure 6A). Mortality in IFNγR$^{-/-}$ mice (Figure 2E) is associated with high levels of persistent replication in both splenocytes and PEC that is not observed in normal mice ($p < 0.0001$, Figure 6B). Consistent with the requirement for the γHV68 RCA protein in virulence in these persistently infected mice, we found that γHV68 RCA protein was also required for efficient persistent replication in IFNγR$^{-/-}$ splenocytes, with γHV68-RCAstop showing ~6.5-fold less persistent replication than wild-type γHV68 (Figure 6B; <1 pfu/100,000 splenocyte equivalents for γHV68-RCAstop compared to ~1 pfu/15,600 splenocyte equivalents for wild-type γHV68, $p = 0.0002$). In the course of evaluating latency in $C3^{-/-}$ mice, we also detected a modest increase in persistent replication of wild-type γHV68 in PEC but not splenocytes of $C3^{-/-}$ mice ($p = 0.038$, Figure 6C). Persistent replication of γHV68-RCAstop in $C3^{-/-}$ PEC was similar to that seen with wild-type γHV68 (Figure 6C), demonstrating that the presence of the γHV68 RCA protein does not alter persistent replication in $C3^{-/-}$ mice. These data suggest that complement plays a role in controlling persistent γHV68 infection and that this effect is countered by the γHV68 RCA protein.

Complement, but Not the γHV68 RCA Protein, Regulates Viral Latency

We next evaluated the role of complement in viral latency. Latency is the presence of reactivatible viral genome within cells in the absence of infectious virus and represents the primary mechanism by which herpesviruses maintain life-long infection. We infected $C3^{-/-}$ mice and control wild-type 129, B6, and 129 x B6 F1 mice with wild-type γHV68 and evaluated the establishment of an ex vivo reactivation from latency 16 days later. The frequency of reactivating cells was comparable in 129, B6, and 129 x B6 F1 mice (~1 in 1514–2016 for PEC, and <1 in 100,000 for splenocytes, Figure 7A). However, the frequency of PEC reactivating from latency was increased in $C3^{-/-}$ mice (Figure 7A, ~1 in 431 for PEC) compared to wild-type mice (129 [$p = 0.0002$], B6 [$p = 0.0012$], and 129 x B6 F1 [$p = 0.0005$]). The frequency of $C3^{-/-}$ splenocytes reactivating from latency (Figure 7A, ~1/48,420 splenocytes) was also increased compared to control wild-type mice (129 [$p = 0.0134$], B6 [$p = 0.0194$], and 129 x B6 F1 [$p = 0.0169$]). The increase in the PEC may be partly explained by an increase in the frequency of $C3^{-/-}$ PEC carrying viral genome (Figure 7B, 129 [$p = 0.016$], B6 [$p = 0.0109$], and 129 x B6 F1 [$p = 0.0254$]), but no statistically significant difference was observed in viral genome bearing splenocytes between $C3^{-/-}$ and control mice (Figure 7B). The differences between the frequency of cells carrying viral genome in vivo and the frequency of cells undergoing reactivation ex vivo is not fully understood, although recent work shows that the immune system does control the efficiency of γHV68 ex vivo reactivation (Tibbetts et al., 2002).

We next evaluated the role of the γHV68 RCA protein in viral latency. The frequencies of PEC and splenocytes reactivating γHV68-RCAstop was comparable to that seen with wild-type γHV68 in B6 mice (Figure 7C). Similar to the results observed with wild-type γHV68, the frequency of cells reactivating γHV68-RCAstop was increased in $C3^{-/-}$ mice (Figure 7D). These data demonstrate that the presence of the γHV68 RCA protein did not alter viral latency or the effect of complement on viral latency. Thus, the effect of C3 on latency (Figure 7A) is not evaded by the γHV68 RCA protein.

Discussion

These studies show that complement is an important host defense against both acute and chronic γ-herpesvirus infection. Moreover, γ-herpesvirus RCA proteins can

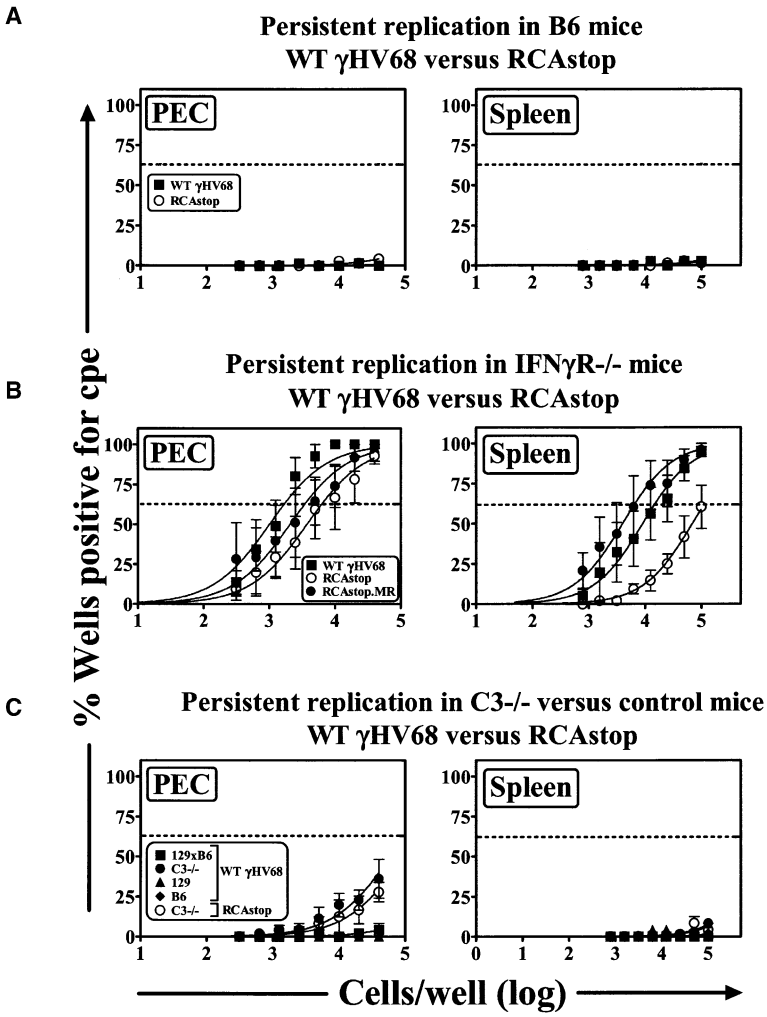

Figure 6. Role of Complement and the γHV68 RCA Protein in Persistent Replication

PEC and splenocytes from wild-type B6 (A), C3$^{-/-}$ (B), and IFNγR$^{-/-}$ (C) mice infected with either wild-type γHV68, γHV68-RCAstop, or γHV68-RCAstop.MR were assessed for the presence of persistently replicating virus at day 16 postinfection. These data (± SEM) are pooled from at least two independent experiments.

play a key role in viral virulence via inhibition of C3-dependent host resistance in vivo. These findings are particularly noteworthy in two major respects. First, while complement has been shown to have a key role in acute viral infection, we made the unexpected observation that complement is important in controlling two components of chronic herpesvirus infection: latency and persistent replication. Second, while the γHV68 RCA protein was remarkably effective at countering the effects of complement during acute infection and persistent replication in immunocompromised IFNγR$^{-/-}$ mice, it was ineffective at countering complement effects on viral latency.

Role of the γHV68 RCA Protein during Acute Infection

γHV68-RCAstop virus replicates normally in cultured fibroblasts in vitro, demonstrating that the protein is not essential. This shows that the γHV68 RCA protein is functionally distinct from poxvirus RCA proteins such as vaccinia B5R protein, which are involved in maturation and cell to cell spread of poxviruses (del Mar et al., 1998; Isaacs et al., 1992b; Martinez-Pomares et al., 1993; Engelstad and Smith, 1993; Wolffe et al., 1993; Takahashi-Nishimaki et al., 1991; Herrera et al., 1998; Mathew et al., 1998). In contrast to the in vitro setting, the γHV68 RCA protein was important for viral replication in vivo in the CNS, although the effects of the γHV68 RCA protein on replication in tissues outside of the CNS was modest. The role of the γHV68 RCA during replication in vivo is largely due to its function as an immune evasion protein since deletion of C3 from the host normalized growth and virulence of γHV68 RCA protein mutant virus.

The 100-fold decrease in virulence of γHV68 RCA mutant virus after i.c. inoculation was associated with decreased viral growth, decreased viral antigen staining, and decreases in the number of infected CNS parenchymal cells at later times of acute infection. Thus, the γHV68 RCA protein is an important virulence factor in this model of acute infection. The γHV68 RCA protein is therefore biologically similar to the vaccinia virus RCA protein VCP and the herpes simplex complement regulator gC which also serve as virulence factors (Adler et al., 2001; Lubinski et al., 1998, 1999; Isaacs et al., 1992a; Friedman et al., 1996; Smith et al., 1997). While the role of the protein was most dramatic in CNS infection, there were also modest effects of the RCA protein mutation on acute replication (Figures 2A and 2C) and persistent replication (Figures 2E and 6B) in the PEC. Therefore,

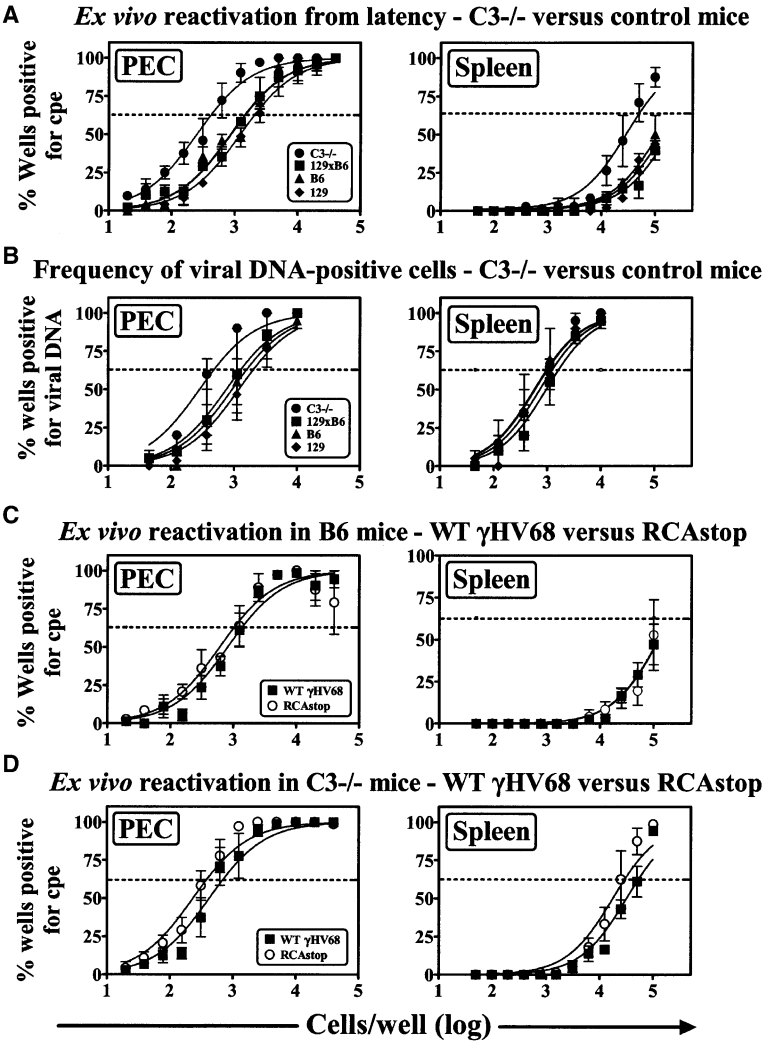

Figure 7. Role of Complement and the γHV68 RCA Protein in γHV68 Reactivation from Latency

(A) Reactivation from latency in PEC and splenocytes from C3$^{-/-}$ mice and wild-type control mice infected with wild-type γHV68.

(B) Frequency of PEC and splenocytes positive for wild-type γHV68 DNA in C3$^{-/-}$ mice compared to wild-type control mice.

(C) Reactivation from latency in PEC and splenocytes from B6 mice infected with either wild-type γHV68 or γHV68-RCAstop.

(D) Reactivation from latency in PEC and splenocytes from C3$^{-/-}$ mice infected with either wild-type γHV68 or γHV68-RCAstop. These data (± SEM) are pooled from at least two independent experiments.

the effects of complement on γHV68 lytic replication are not specific for the CNS.

The Mechanism of Action of the γHV68 RCA Protein In Vivo

The γHV68 RCA protein functions in vivo by regulating complement, since the attenuated phenotype of γHV68-RCAstop is reversed by deletion of host C3, and this reversal is specific for both C3 and the γHV68 RCA protein. This is an important finding since RCA proteins may have roles independent of complement regulation (del Mar et al., 1998; Isaacs et al., 1992b; Martinez-Pomares et al., 1993; Engelstad and Smith, 1993; Wolffe et al., 1993; Takahashi-Nishimaki et al., 1991; Herrera et al., 1998; Mathew et al., 1998; Laquerre et al., 1998; Sears et al., 1991; Lubinski et al., 1998). For example, the vaccinia virus VCP protein also binds heparan sulfate and is involved in interactions with human endothelial cells (Smith et al., 2000). The γHV68 RCA protein is very effective in this immune evasion role since host C3 does

Table 1. Sequences of Primers Used in This Study

Amplified DNA/Primer	Size (bp) of Product	Sense Primer (5′-3′)	Antisense Primer (5′-3′)
RCAstop:lit28 fragment 1	2098	GCCACCT*ACGCGT*CACGCATG[a]	TAGCTC*GTTAAC*TTCAGCGGAGCTGCAGGGTG[a]
RCAstop:lit28 fragment 2	303	TAGCTGAA*GTTAAC*GGGTTCACACCAGGCGC[a]	TTATGTCA*CATATG*GGAGATTCCCCACTCC[a]
γHV68-RCAstop outer primers	643	(primer A) AAGTTCAATTCCTTGTGCC	(primer B) GGTTGGCCCATATAGCTTCAGATT
γHV68-RCAstop inner primers	328	(primer C) GCAGCTCCGCTG**AGGTTA**[b]	(primer D) TCGGGAGGTGCACACTTTT
γHV68-RCAstop.MR primer	NA	GCAGCTCCGCTG**TAGGCC**[b]	NA
γHV68 *gene 72*	133	AAAGTCTGGATACCCTCTCG	GAAGATTTTTGGGTGACAGAG

[a] The italicized DNA sequence represents introduced restriction enzyme sites. The underlined sequence represents sequence not found in the template DNA being amplified.

[b] The bold sequence represents unique sequence in γHV68-RCAstop DNA (primer C) and γHV68-RCAstop.MR.

not regulate the virulence of wild-type γHV68 which expresses the RCA protein but does regulate the virulence of γHV68RCA.stop which lacks the RCA protein.

The mechanism by which complement plays a role in protecting the CNS from viral infection is not known. The presence of the viral RCA protein in the meninges and on the surface of infected cells in the brain raises the possibility that the viral RCA protein is protecting infected cells from complement-mediated lysis. However, both γHV68 and HVS express soluble forms of the RCA protein (Kapadia et al., 1999; Albrecht and Fleckenstein, 1992), suggesting that inhibition of complement activation in the fluid phase could also be important for host resistance. The relative contribution of complement regulation in the fluid phase versus at the cell or virion surface will have to be examined using viruses that are unable to express either the soluble or membrane-bound isoforms of the γHV68 RCA protein.

Since the soluble γHV68 RCA protein isoform can regulate C3 deposition via both classical and alternative pathways (Kapadia et al., 1999), it was possible that both the classical and alternative pathways could play a role in γHV68 infection of the CNS. However, data showing that deletion of factor B, which is critical for the alternative pathway, does not reverse the attenuation of a γHV68 RCA protein mutant suggests that the alternative pathway is not the primary target of the γHV68 RCA protein during acute infection of the CNS. This suggests that the classical and/or the mannose-binding lectin complement pathways are important for host resistance to γHV68 (Kapadia et al., 1999).

The general importance of complement regulation during DNA virus infection is underlined by comparison of the results presented here with studies of the HSV gC-1 protein. While this protein has complement-independent functions, including in binding heparan sulfate (Laquerre et al., 1998) and regulating infection of polarized epithelial cells (Sears et al., 1991; Lubinski et al., 1998), it plays an important role in vivo by virtue of regulating complement (Lubinski et al., 1998, 1999; Friedman et al., 1996). The gC protein is not structurally homologous to the γHV68 RCA protein. Thus, both α- and γ-herpesviruses have evolved mechanisms for inhibiting C3 dependent immunity, but have done so via convergent evolution.

Role of the γHV68 RCA Protein and Complement during Persistent Replication

One of the more surprising findings of this study was the role of complement and complement evasion during chronic (latent and persistent) γHV68 infection. One important component of chronic γ-herpesvirus infection is persistent replication. At day 16, acute virus in the PEC and splenocytes is cleared by wild-type mice (Weck et al., 1996, 1999a, 1999b; Clambey et al., 2000; Van Dyk et al., 2000). However, in immunocompromised mice, including those lacking IFNγ or the IFNγR, γHV68 persistently replicates at high levels (Tibbetts et al., 2002; Dal Canto et al., 2000; Gangappa et al., 2002). We found that the γHV68 RCA protein was important for efficient persistent replication in IFNγR$^{-/-}$ mice. The physiologic importance of this is shown by the decreased virulence of a γHV68 RCA protein mutant virus in these mice (Figure 2E). The importance of complement in the control of persistent replication is confirmed by the finding of persistent replication of wild-type γHV68 in C3$^{-/-}$ mice.

The mechanism(s) responsible for the roles of complement and the γHV68 RCA protein in persistent replication are not clear. Either increased reactivation from latency in the absence of complement (see below) or increased replication of wild-type γHV68 compared to γHV68-RCAstop, as was observed during acute replication, could contribute to enhanced persistent replication in immunocompromised mice. It is also possible that complement activation during virus infection is necessary for induction of immune responses that eliminate persistent replication.

Role of Complement in γ-Herpesvirus Latency

We found an increased number of latently infected cells in C3$^{-/-}$ mice using an ex vivo reactivation assay, demonstrating that complement plays a role in γ-herpesvirus latency. The lack of effect of the γHV68 RCA protein, despite its importance during acute infection and persistent replication in immunocompromised mice, was particularly striking. One explanation for this is that the γHV68 RCA protein is a lytic cycle late protein and may therefore not be expressed by latently infected cells (Virgin et al., 1999; Kapadia et al., 1999). However, another lytic cycle protein, the viral cyclin, has prominent effects on viral latency (Gangappa et al., 2002; Hoge et al., 2000; Van Dyk et al., 2000). If persistent replication contributes to latency, it is possible that complement-dependant regulation of reactivation from latency may be due to effects on viral replication. It is also possible that the effects of complement on viral latency are indirect. Since B cells are critical for regulating γHV68 latency (Weck et al., 1999a), a possible interaction between B cells and complement in the regulation of latency is an attractive possibility. Consistent with this hypothesis, passive transfer of γHV68-specific antibody to latently infected B cell$^{-/-}$ mice can efficiently inhibit wild-type γHV68 reactivation from latency (Gangappa et al., submitted; Kim et al., 2002). It is tempting to speculate that the effects of complement on latency are secondary to the need for complement for B cell switching or antigen presentation to T cells (Carroll, 2000; Luxembourg and Cooper, 1994; Cherukuri et al., 2001; Nielsen et al., 2000). Based on the data we present here, it would also be interesting to assess the relationship between complement-deficient patients and EBV or KSHV infection.

Experimental Procedures

Virus, Viral Assays, and Tissue Culture

γHV68 clone WUMS (ATCC VR1465) was passaged and grown in NIH 3T12 cells (Weck et al., 1996, 1997; Kapadia et al., 1999). Murine embryo fibroblasts (MEF) were obtained from C57Bl/6 (B6) mice (Weck et al., 1996). To assess viral growth in cultured cells, NIH 3T12 cells were infected with mutant or wild-type γHV68 viruses, and viral titer was measured (Clambey et al., 2000; Van Dyk et al., 2000). The level of detection for all plaque assays was 50 pfu (1.7 in $\log_{10}$).

Generation of Recombinant γHV68 Viruses

The γHV68-RCAstop targeting vector (RCAstop:lit28) was generated using PCR mutagenesis from a genomic subclone plasmid (RCA:lit28)

containing the 7948 bp BglII-XhoI fragment (bp 5362–13,311) of γHV68 cloned into Litmus 28 (New England Biolabs, Beverly, MA). γHV68 sequence from the MluI restriction site (bp 7949 of the γHV68 genome) to bp 10,031 was amplified (fragment 1, primers in Table 1), while inserting two translational stop codons and a frameshift in the γHV68 RCA ORF. A unique HpaI restriction site was incorporated for Southern blot analysis. γHV68 sequence from bp 10,031 to NdeI restriction site (bp 10,309) was amplified (fragment 2, primers in Table 1). Fragments 1 and 2 were digested with MluI/HpaI and HpaI/NdeI, respectively, cloned into RCA:lit28 to generate RCAstop:lit28, and sequenced (Sambrook et al., 1989; Clambey et al., 2000; Van Dyk et al., 2000).

The γHV68-RCAstop virus was generated by cotransfecting RCAstop:lit28 with wild-type γHV68 genomic DNA (Clambey et al., 2000; Van Dyk et al., 2000; van Berkel et al., 2002; Gangappa et al., 2002). Plaques were screened by nested PCR using outer primers A and B (which amplify both wild-type γHV68 and γHV68-RCAstop DNA sequence, Table 1) and the inner primers C and D (specific for γHV68-RCAstop, Table 1). The marker rescue virus (γHV68-RCAstop.MR) was generated by cotransfecting RCA:lit28 plasmid with γHV68-RCAstop viral DNA (Clambey et al., 2000; Van Dyk et al., 2000; van Berkel et al., 2002). γHV68-RCAstop.MR plaques were selected by nested PCR using outer primers A and B and inner primer C together with a γHV68-RCAstop.MR-specific primer (Table 1). PCR conditions for γHV68-RCAstop round 1 were: (1) denaturation at 95°C, 5′; (2) 35 cycles, 95°C, 1′; 54°C, 1′; 72°C, 2′; (3) extension at 72°C, 5′; and (4) 4°C hold. PCR conditions for γHV68-RCAstop round 2 were: (1) denaturation at 95°C, 5′; (2) 35 cycles: 95°C, 1′; 51°C, 1′; 72°C, 1′; (3) extension at 72°C, 5′; and (4) 4°C hold.

All viruses were plaque purified to homogeneity and confirmed by nested PCR, Southern blot, and Western blot analyses (Clambey et al., 2000; Van Dyk et al., 2000; Kapadia et al., 1999; van Berkel et al., 2002). Twenty individual γHV68-RCAstop and γHV68-RCAstop.MR plaques were assessed for correct integration of the mutation by probing HpaI+SpeI digested viral DNA with probe A (Figure 1A). Analysis of the left end of the γHV68 genome for deletions was performed using probe B (Clambey et al., 2000; Van Dyk et al., 2000) (Figure 1A). Western blot analysis was performed to detect soluble and membrane-associated γHV68 RCA protein (Kapadia et al., 1999). To detect truncated forms of the γHV68 RCA protein, supernatants from virally infected cells were concentrated using Centriprep 3000 MW cut-off concentrators (Millipore Corporation, Bedford, MA).

Real-Time RT-PCR Analysis of Mutant and WT γHV68 Viruses

We isolated RNA from infected cells and performed real-time PCR analyses to assess viral transcript levels (van Berkel et al., 2002). To quantitate viral transcripts, dilutions of plasmids containing genes *M4*, *gene 6*, and *gene 72* were always run in parallel with cDNA from infected cells (dilutions ranged from 10^9–10^1 plasmid copies per reaction). PCR primers for genes *M4* and *gene 6* used for real-time PCR analysis were as described (van Berkel et al., 2002). PCR primer sequences for *gene 72* are shown in Table 1.

Mice, Infections, and Organ Harvests

All mice were sex and age matched and infected between 7–9 weeks of age unless otherwise stated. C57Bl/6 (B6), C57BL/6J-*Rag1*^{tm1Mom} (RAG1$^{-/-}$ mice), and 129 x B6 F1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). C3$^{-/-}$ mice (on a mixed 129 x B6 background) were originally obtained from Harvey Colten (Kapadia et al., 1999). CD-1 outbred mice with litters (at least 10 pups) were obtained from Charles River Laboratories (Wilmington, MA). Mice were bred and maintained at Washington University School of Medicine in accordance with all federal and university policies. Adult mice were infected by the i.p. route of infection with 1–5 $\times$ 10^6 pfu in 0.5 ml of supplemented DMEM medium unless otherwise noted. Intracranial (i.c.) inoculations of weanling mice were performed as described using 100 pfu unless otherwise noted (van Berkel et al., 2002). Viral titers in organs were determined by plaque assay (Clambey et al., 2000; Van Dyk et al., 2000).

Immunofluorescence for Viral Antigen

Brain halves were fixed, and immunofluorescence was performed on paraffin-embedded sagittal sections (Weck et al., 1997; Dal Canto et al., 2000; van Berkel et al., 2002). The number of viral antigen-positive cells in the cortex was quantitated in two sections per mouse by three investigators counting in a blinded manner. For 5 days postinfection, six mice infected with wild-type γHV68 and six mice infected with γHV68-RCAstop were examined. For mice harvested 7 days postinfection, thirteen mice infected with wild-type γHV68 and eight mice infected with γHV68-RCAstop were examined.

Ex Vivo Analysis of γHV68 Latency and Reactivation

The frequency of splenocytes or peritoneal cells that reactivate from latency ex vivo was determined using a limiting dilution reactivation assay (Weck et al., 1996, 1999a, 1999b; Van Dyk et al., 2000; Clambey et al., 2000; van Berkel et al., 2002). Mechanical disrupted cells contained <1% live cells, and thus the presence of preformed virus (persistent replication) was detected by serial dilution of mechanically disrupted cells (Weck et al., 1996, 1999a, 1999b; Van Dyk et al., 2000; Clambey et al., 2000). The frequency of peritoneal cells or splenocytes harboring γHV68 genome was determined using a limiting dilution nested PCR assay (Weck et al., 1996, 1999a, 1999b; Van Dyk et al., 2000; Clambey et al., 2000; van Berkel et al., 2002). There were no false positive PCR reactions in the data in this report, and all assays demonstrated approximately one copy sensitivity for plasmid DNA. For all experiments, data on latency, persistent replication, and the frequency of genome-bearing cells was pooled from at least three experiments with five mice per group per experiment.

Statistical Analysis

All data was analyzed using GraphPad Prism program (GraphPad Software, San Diego, CA) as previously described (Weck et al., 1996, 1999a, 1999b; Van Dyk et al., 2000; Clambey et al., 2000; van Berkel et al., 2002). Frequencies of reactivation and genome-positive cells were obtained from cell numbers at which 63% of wells scored positive for either viral CPE or for presence of viral genome based on Poisson distribution. The data were then subjected to nonlinear regression analysis to obtain the single-cell frequency for each limiting dilution analysis.

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